REMARKS

Applicants respectfully request reconsideration of the present application in view of the foregoing amendments and the following comments.

Claims 1-4, 6, 15, 16, 20, 21, 34, and 35 are amended presently. Additionally, claims 41 and 42 have been added. Applicants request that the examiner enter the above claim amendments and new claims as no new matter has been added. In particular, exemplary support for new claim 41 may be found in the original claim 1 as well as the specification on page 7, lines 28-30. Support for new claim 42 may also be found in original claim 1 and page 4, lines 25-29. Finally, support for the amendments to claim 1 may be found in the specification on page 1, line 34 - page 2, line 8, and page 4, lines 16-20. Upon entry of this response, claims 1-42 will be pending.

I. Rejection under 35 USC §112

A. Rejection for alleged lack of written-description support

Claims 1-29, 31-40 are rejected under 35 U.S.C. §112, first paragraph, for failing to comply with the written-description requirement. The examiner argues that "the instant specification teaches the construction of two plasmid replicons" but does not teach the structural/functional characteristics of all of the potential variations of these vectors. Applicants respectfully disagree.

Applicants have amended claim 1 to cover a vector comprising:

a. lactic acid bacterial DNA;

b. a gene coding for an amber suppressor which is a tRNA comprising the CUA anticodon; and

c. a replicon making the vector capable of replicating in a lactic acid bacterium;

but lacking an antibiotic resistance gene.

The specification describes in detail each of these structural characteristics. For example, the specification describes the claimed vector as lacking an antibiotic resistance gene on page 1, line 34 - page 2, line 8, and page 4, lines 16-20. The specification also describes sources of the DNA material for the invention, page 6, lines 15-19. Additionally, the specification also discloses the various mutations of the amber suppressor suitable for the invention, page 6, line 28-33 and provides various

examples of suitable suppressors on page 8, line 12-34 of the specification. Finally, the specification provides examples of replicons capable of replicating in a lactic acid bacterium on page 9, lines 25-30, along with the size of the claimed vectors (page 10, lines 12-15). Furthermore, one of skill in the art would know how to select a replicon capable of replicating in a desired bacterial strain. See Bolivar et al. 1977, Exhibit A, and Pedersen *et al.* 1994, submitted as IDS, A17, Exhibit B.

Accordingly, applicants' specification provides a clear indication that applicants, at the time their application was filed, possessed the claimed invention.

B. Rejection for alleged non-enablement

Claims 1-40 also stand rejected under Section §112 for allegedly failing to satisfy the enablement requirement. The examiner found that the claims cover a broad genus of recombinant vectors and, hence, that the skilled person would not be able to determine the additional vectors that would meet the requirements of claim 1. Applicants respectfully disagree.

As explained above, applicants have amended claim 1 to clearly describe the structure of the claimed genus. Thus, one of skill in the art would easily be able to obtain the claimed vectors using only routine experimentation. Additionally, a person skilled in the art would know how to test a particular vector and would easily recognize whether this vector falls within the functional characteristics of the claimed vectors, i.e. being stably maintained in an industrial useful strain, substantially not causing growth inhibition and reducing acidification in a broad range of host strains. Specification page 4, lines 16-20 and page 4, line 31 – page 5, line 13.

Furthermore, as requested by the examiner, applicants have amended claim 15 to remove the "mutants, variants and derivatives" language from the claims. Given the specification's description and the clearly defined scope of the present claims, both set forth above, one of skill in the art would be able to practice the claimed invention.

Finally, per the examiner's request, applicants have attached a declaration by Jan Skouv that states that the biological materials necessary to practice the invention are deposited under the Budapest Treaty and are available for use. See attached Exhibit C.

Therefore, one of skill in the art is able to practice the present invention without undue experimentation, hence, the present invention is enabled and that this rejection should be withdrawn.

C. Rejection for alleged indefiniteness

Claims 1-40 are rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to point out and distinctly claim the subject matter that the applicants regard as their invention. Accordingly, applicants have amended the claims per the examiner's requests.

Specifically, applicants amended claim 1 to clarify the structural characteristics of the claimed vector i.e., a vector "comprising (a) lactic acid bacterial DNA, (b) a gene coding for an amber suppressor that is a tRNA comprising the CUA anticodon, and (c) a replicon making the vector capable of replicating in a lactic acid bacterium." Also, claim 41(ii), formerly claim 1(ii), has been amended to specify that the DSM 12109 strain is used to compare the functional properties of the claimed vector against the functional properties of the parent strain and to clarify the acidification rate variation allowed between the parent strain and the strain containing the claimed vector. Additionally, claims 4, 6, 10, and 20 were amended to recite "obtained from" rather than "derived from," claim 6 also was amended to cover "a heterologous promoter" rather than "a promoter not naturally related to the gene," claim 15 was amended to remove "mutants, derivatives, or variants," claim 16 was amended to recite "further comprises," claim 21 was amended to specify that the gene product is "a part of the nisin synthesis pathway or leading to nisin resistance" and claim 34 was amended to recite "per gram composition." Finally, claim 35 was amended to comport with proper claim structure and language and to clarify that claim 35 covers the "use of a the composition ... as a starter culture in the preparation of a product."

Separately, the examiner found that there was not a clear antecedent basis for the phrase "the gene product" as used in claims 16 and 18. Applicants respectfully disagree. Amended claim 16 now provides proper antecedent basis for the phrase "the gene product" as used in dependant claim 18.

The examiner also found that claims 32 and 33 were vague and indefinite because they recite the phrase "pure culture." Applicants argue that "the phrase "pure culture" is clearly defined on page 12, lines 5-6 of the specification, as a culture containing biomass of one single isolate of a lactic acid bacterial species, *i.e.*, a clone originating in principle from one cell.

Applicants believe that the present claims clearly define the subject matter of the present invention. Therefore, applicants argue that the above rejections should be withdrawn and the present claims allowed.

II. Rejection Under 35 USC §101

Claim 35 is rejected under 35 U.S.C. §101 because it is not a proper process claim. As explained above, applicants have amended claim 35 and believe that it is now written in proper claim format. Therefore, applicants believe that the present rejection should be withdrawn and the claim allowed.

III. Rejection Under 35 USC §102

Claims 1-4, 7, 9-16, 22-24, 28-29, 31-33, and 36 are rejected for anticipation by Dickely *et al.* Applicants respectfully disagree.

At the outset, applicants would emphasize that the specification defines a "food-grade vector" as a recombinant vector that "consist[s] essentially of lactic acid bacterial DNA" (page 2, lines 11 & 12) and that lacks an antibiotic resistance gene (see page 4, lines 2 & 3). By the same token, amended claim 1 recites a vector that lacks an antibiotic-resistance gene but that does comprise (a) DNA from lactic acid bacterial origin, (b) a gene coding for an amber suppressor that is a tRNA comprising the CUA anticodon, and (c) a replicon that renders the vector capable of replicating in a lactic acid bacterium. In contrast, both of the cloning vectors disclosed by Dickely *et al.* (1995), pAK89 and pAK89.1, contain a gene coding for erythromycin resistance.

Furthermore, the Dickely reference does not anticipate a recombinant vector "consisting essentially of lactic acid bacterial DNA" (claim 42). The Dickely vectors, pAK89 and pAK89.1, contain not only a gene coding for erythromycin resistance but also DNA from *E. coli*, along with DNA from a lactic acid bacterium (see Dickely's Table 3). As evidenced by the specification, *e.g.*, at page 1, lines 23-32 and page 2, lines 10-12, the presence of the *E. coli* DNA disqualifies the Dickely vectors for food use; that is, they are not "food grade." It is apparent, therefore, that a vector "consisting essentially of" the constituents recited in claim 42 would not include DNA, such as *E. coli* DNA, that affects so material a characteristic as its "food grade" quality.

For these reasons alone, claim 1 and claim 42 are separately patentable over the prior art illustrated by Dickely *et al.* More generally, applicants would emphasize that the presently claimed invention distinguishes over the art because the recited cloning system is stably maintained and useful in any industrial lactic acid bacterial strain, in contrast to conventional cloning systems. In

addition, the claimed cloning system does not cause growth inhibition and reduces acidification in a broad range of host strains, especially in industrial useful lactic acid bacteria. These characteristics make applicants' claimed vectors useful in the manufacturing of food and feed products.

In fact, at the time of filing the art taught away from the use of an amber suppressor, since it was conventional wisdom that the amber suppressor would cause growth inhibition since this was the result seen with an ochre suppressor. Furthermore, at the time of filing for the present application, the whole lactic acid bacteria genome was not known; hence, one would not know how many amber stop codons (UAG) an amber suppressor would recognize in a cell, compared to an ochre suppressor.

In summary, the present claims do not read on the cloning vectors taught by the cited publication, and so the subject matter of the claims is novel over the art at the time of filing. Applicants therefore submit that this rejection should be withdrawn.

IV. Priority

Applicants submit, herewith, a new application data sheet, Exhibit D, which properly reflects the present application's priority information. Additionally, attached as Exhibit E is a copy of PCT/IB/304 indicating that the International Bureau received a copy of each of the priority applications on May 12, 1999. Therefore, applicants do not believe they are required to submit additional certified copies.

CONCLUSION

Applicants believe that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested. The examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, applicants hereby petition for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date 30 December 2004

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CC/NUMBER 47
NOVEMBER 19, 1990

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Bolivar F, Rodriguez R L, Greene P J, Betlach M C, Heyneker H L, Boyer H W, Crosa J H & Falkow S. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* 2:95-113, 1977.
[Department of Biochemistry and Biophysics, University of California, San Francisco, CA]

The plasmid pBR322 was one of the first certified EK2 multipurpose cloning vectors to be available for the efficient cloning and propagation of recombinant molecules in *Escherichia coli*. This DNA molecule has been extensively used because of its simplicity and the availability of its nucleotide sequence since the early days. Today, pBR-22 is still used as a molecular cloning vehicle, although more advanced vectors have been developed from it.[1] [The *SCI*® indicates that this paper has been cited in more than 3,395 publications, making it the most-cited paper from this journal.]

Multipurpose Tools in Molecular Biology

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June 13, 1990

Significant advances in scientific endeavors are always accomplished faster if work is supported by efficient tools and methodologies. The inability to concretize ideas in scientific research, due to methodology limitations, may be quite frustrating. Therefore, methods and research tools are usually subject to evolution themselves, so their refinement becomes a strategic aspect of scientific progress. The story of the design, construction, and characterization of the multipurpose cloning vehicle, the plasmid pBR322, is an example of this notion.

In the early days of molecular cloning, plasmid vectors were poorly characterized: They exhibited high molecular weights and were devoid of convenient cloning sites. Little was known about their intrinsic features, such as stability, coding properties, and functions. This was the scenario when I joined Herbert Boyer's group at the University of California, San Francisco, in 1973. This group was composed of several bright scientists from different countries working hard on the isolation and manipulation of specific genes. However, progress in the work was slow, I believe mainly because of limited tools, particularly the cloning vectors and low purity restriction endonucleases and T4 DNA ligase. Inevitably, some of the members of the laboratory decided to work towards the development of new and more efficient tools.

Initially, Herb Boyer was not very keen on the idea of constructing a new vector because we already had pMB9, so most of this work was done during our "spare time." Nevertheless, when pBR322 was constructed, he became a strong supporter of the new cloning vehicle and made it readily available to the scientific community. The plasmid was distributed to over 300 laboratories all over the world during those early days. I strongly believe that this was one of the reasons scientists adopted pBR322 as a member of their labs.[2] Convenience of cloning pBR322 by inactivation of antibiotic resistance genes, and various unique restriction sites, offered a simple way to design experiments and a rapid analysis of results, rendering pBR322 quite superior to its parental plasmid, pMB9. Moreover, safety of the cloning procedures was then in the minds of millions, and pBR322 was built with a highly diminished ability to propagate outside laboratory cells. In fact, this was the first example of an EK2 system to be certified as safe according to the National Institutes of Health recombinant DNA guidelines. Finally, the elucidation of the complete nucleotide sequence of pBR322 two years later by Greg Sutcliffe at Wally Gilbert's lab[3] strongly contributed to its popularity, yielding experimental design more versatile and precise. The following quotation summarizes the impact of pBR322 in the late 1970s and early 1980s: "It was, in short, a compact dream machine of a plasmid."[4]

After more than a decade, pBR322 is still being used in a large variety of ways. Most importantly, this multipurpose cloning vector has been used as the parental plasmid of many specialized vectors utilized today, not only for *Escherichia coli*, but also for interspecies shuttle vectors. Its origin of replication, coding regions, structural features, and functional capabilities have been so extensively studied that parts of pBR322 continue to be used as components for the development of new plasmids.[1] The paper describing the construction of pBR322 became a *Citation Classic* long ago.[5] However, it is more meaningful to me that the original paper is not referred to anymore in a large number of papers, perhaps having achieved wide recognition.

Finally, as my good friend Pierre Prentki quoted to me once, "...a myth starts when the same character belongs to more than one story." pBR322 has now transcended the walls of molecular biology laboratories. Its restriction map is exhibited in watches distributed by Boehringer Mannheim, and even a love story has been published regarding one of the derivatives of pBR322: "...One day, a messenger of King Pebearius CCCXXII came through Coliborough upon Tween and told the villagers about the great misfortune that had befallen the king. A strange blue light had been seen in the palace one night, and the next morning Princess Clonia had not appeared for lunch."[6]

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14-12

Mol Gen Genet (1994) 244:374–382

ORIGINAL PAPER

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Genetic analysis of the minimal replicon of the *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* citrate plasmid

Received: 8 October 1993 / Accepted: 15 February 1994

Abstract Using a combination of mutagenesis with the transposon γδ and polymerase chain reaction subcloning, the essential elements of the replication region of the *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* citrate plasmid have been identified. An open reading frame, coding for a protein with homology to Rep proteins from other *Lactococcus* plasmids, is essential. This protein is *trans*-acting and could not be replaced by the Rep protein from another *Lactococcus* plasmid. A second open reading frame immediately downstream from the first could be removed or inactivated with no apparent effect on plasmid replication. A region containing two 10 bp direct repeats and three tandem repeats of a 22 bp sequence, immediately upstream of the essential open reading frame, is also essential and probably includes the origin of replication. A 181-bp DNA fragment containing this region was sufficient to allow replication in *Lactococcus* if the *trans*-acting protein was provided on another replicon. Single-stranded replication intermediates could not be detected, suggesting that the citrate plasmid uses theta replication rather than rolling-circle replication.

Key words *Lactococcus* · Citrate plasmid
Minimal replicon · *repB*

Introduction

Lactococcus lactis subsp. *lactis* biovar *diacetylactis* is an important component of mesophilic starter cultures used to produce buttermilk and cheeses with small holes (e.g. Danbo, Havarti, and Gouda). These bacteria ferment citrate and produce important flavor compounds such as diacetyl (Marshall 1987).

The ability to ferment citrate is dependent on the presence of a plasmid (Kempler and McKay 1981) containing the citrate permease gene (David et al. 1990). This plasmid is highly conserved in *L. lactis* subsp. *lactis* biovar *diacetylactis* (Gasson and Davies 1984). Identical sizes and restriction maps have been found for the citrate plasmid from a number of strains (Kempler and McKay 1981).

The replication region of pSL2, the citrate plasmid of *L. lactis* subsp. *lactis* biovar *diacetylactis* Bu2, has been identified and the DNA sequence determined by Jahns et al. (1991). This region contains two open reading frames (Fig. 1). We have found that the potential translation product of one of these open reading frames (Orf 1) has high homology to the RepB protein of pCI305 (Hayes et al. 1991) and pWVO2 (Kiewiet et al. 1993a) and the replication protein of other *Lactococcus* plasmids (Horng et al. 1991; Lucey et al. 1993; von Wright and Räty 1993) and designate this gene *repB* for this reason. Immediately outside the proposed minimal replicon (Jahns et al. 1991) is a region with small repeated sequences resembling the origin of replication of other *Lactococcus* plasmids (Hayes et al. 1991; Horng et al. 1991; Kiewiet et al. 1993a; Lucey et al. 1993; von Wright and Räty 1993).

We have used a combination of mutagenesis and polymerase chain reaction (PCR) subcloning to identify the essential elements in the replication region of the citrate plasmid of a wild-type *L. lactis* subsp. *lactis* biovar *diacetylactis* isolated from a commercial mixed-strain mesophilic starter culture. Similarities to the replication region of five other *Lactococcus* plasmids and significant differences from the published sequence of pSL2 (Jahns et al. 1991) are discussed.

Communicated by B. J. Kilbey

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Table 1 Plasmids

Plasmid	Description[a]	Source/reference
pCT1138	Citrate plasmid of DB1138	This study
pVA891	Ery[R]; replicon-cloning vector	Macrina et al. (1983)
pIC19H	Amp[R]; *Escherichia coli* cloning vector	Marsh et al. (1984)
pCI3340	Cam[R]; *E. coli-Lactococcus* shuttle vector containing the pCI305 replication region	Hayes et al. (1990)
pNZ18	Cam[R]; broad host range vector	de Vos (1987)
pAK49	Ery[R]; pCT1138 cloned in pVA891	This study
pMP101-pMP200	Ery[R]; pAK49 containing transposon γδ at various locations	This study; see Fig. 1
pKR45-pKR54	Ery[R]; pVA891 containing various PCR fragments from the citrate plasmid replication region	This study; see Fig. 1
pAK58	Ery[R]; pVA891 containing a PCR fragment with an amber mutation in the citrate plasmid *repB* gene	This study; see Fig. 1
pAK107	Amp[R]; pIC19H containing the PCR product produced with primer 5 and γδ-3	This study; see Fig. 1
pKR44	Amp[R]; pIC19H containing the PCR product produced with primer 2 and primer 4	This study; see Fig. 1
pMP44	Cam[R]; pCI3340 containing the citrate plasmid *repB* on a *Cla*I-*Eco*RI fragment from pKR44	This study

[a] Antibiotic resistance markers are designated: Ery[R], erythromycin resistance; Amp[R], ampicillin resistance; and Cam[R], chloramphenicol resistance

Materials and methods

Bacterial strains, plasmids and media

DB1138 is a wild-type *L. lactis* subsp. *lactis* biovar *diacetylactis* and was isolated from a commercial mixed-strain mesophilic starter culture (Flora Danica Normal, Chr. Hansen's Laboratorium, Hørsholm, Denmark) by plating dilutions on modified Leesment's medium (Vogensen et al. 1987; Johansen and Kibenich 1992a). This strain ferments citrate and was differentiated from *Leuconostoc* by virtue of its sensitivity to vancomycin (Orberg and Sandine 1984), inability to produce gas in MRS medium and inability to cleave X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside). The pattern of carbohydrates fermented, tested with the API 50 CH system (API S.A. System, Montalieu-Vercieu, France) confirmed that DB1138 was a member of the genus *Lactococcus*.

MG1363 is a plasmid-free *L. lactis* strain (Gasson 1983). FD100 is MG1363 containing an ochre suppressor (F. Dickely, personal communication). *Escherichia coli* DH5α [*supE*44 *lac*ΔU169 *hsd*R17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1 Φ80*lac*ZΔM15] (Hanahan 1983) was used for cloning. *E. coli* strains ER [F$^+$ *asnA*31 *asnB*32 *relA*1 *spoT*1 *thi*-1] (von Meyenburg et al. 1979) and R594 [F$^-$*rpsL*179 *galK*2 *galT*22 *lac*-3350] (Campbell 1965) were used for γδ mutagenesis. Plasmids are described in Table 1.

Lactococcus strains were routinely grown at 30° C in M17 medium (Terzaghi and Sandine 1975) containing glucose instead of lactose as carbon source. *E. coli* strains were grown in LB medium (Miller 1972) at 37° C. Plates contained 1.2% agar. Antibiotics were used at the following concentrations: for *E. coli*; 100 μg/ml streptomycin, 250 μg/ml erythromycin (Ery), and 50 μg/ml ampicillin; for *Lactococcus*; 1 μg/ml erythromycin, and 5 μg/ml chloramphenicol (Cam).

Plasmid preparations and transformations

Digestion with restriction enzymes, ligation, plasmid preparations from *E. coli* and transformation of *E. coli* were essentially as described by Sambrook et al. (1989). Plasmid DNA for sequencing and electroporation was prepared with the Qiagen plasmid kit (Diagen, Dusseldorf, Germany).

Large scale plasmid preparation from *Lactococcus* was as described by Anderson and McKay (1983). Small-scale preparation was by alkaline lysis as described by Israelsen and Hansen (1993) with the following modifications: cells (2 ml) were harvested from fresh overnight cultures, sucrose was used at 20% w/v, and potassium acetate was used instead of sodium acetate. Reagent volumes were scaled down proportionately.

Plasmids were introduced into MG1363 by electroporation of glycine-grown competent cells (Holo and Nes 1989). In experiments to test the ability of plasmids to replicate in MG1363, 100 ng of pNZ18 (Cam[R]) was mixed with 500 ng of the pVA891 derivative (Ery[R]) to be tested. Plasmids were co-precipitated with ethanol and resuspended in 10 μl H_2O. After electroporation, cells were plated on erythromycin and on chloramphenicol. Results on erythromycin were only considered valid if a high frequency of Cam[R] colonies appeared.

Construction of pAK49

A large scale plasmid preparation of DB1138 was digested with *Bam*HI and ligated to pVA891 that had been digested with *Bam*HI and phosphatase treated. Transformation of DH5α gave a variety of clones, one of which was designated pAK49 and contains the entire citrate plasmid, pCT1138, inserted in pVA891.

Mutagenesis with transposon γδ

Plasmid pAK49 was transformed into strain ER, producing strain MP8. Liquid matings between MP8 and R594 were done (Miller

1972) and transconjugants selected on LB plates containing erythromycin and streptomycin. Analysis of 100 transconjugants from five independent matings (pMP101–pMP200) revealed the presence of γδ in a variety of locations in pAK49. The positions of γδ in mutants with inserts in the replication region were determined by DNA sequencing (see below) and are indicated in Fig. 1.

Polymerase chain reactions

Oligonucleotide primers corresponding to nucleotides 611–631 (primer 1; TGAATTCAGAGGTTTGATGACTTTGACC) and 837–857 (primer 2; TGAATTCAATCGATGTACACAGTACGAC) and complementary to nucleotides 2070–2090 (primer 3: AGAATTCTGGCAGATATTGTTCAATGGC), 2341–2361 (primer 4; GGAATTCCTAACAAAAGACTATTAACGC) and 854–875 (primer 5: AGAATTCAGTACACAAATACAAAAGTCA) of Jahns et al. (1991) were synthesized by M. Blom Sørensen (Carlsberg Laboratories, Copenhagen, Denmark) on an Applied Biosystem 380A synthesizer using the manufacturer's recommended protocol. Primer γδ-3 (AGAATTCGTTCCATTGGCCCTCAAACCCC), corresponding to the first and last 25 bp of γδ was also synthesized. All six primers contained an *Eco*RI site at the 5' end to facilitate cloning. Mismatch primers (amber 1 and amber 2) with two mismatches were also synthesized. The sequence of these is indicated in Fig. 2. Polymerase chain reactions were done with 35 cycles of 90 s denaturation at 94° C, 60 s annealing at 55° C and 60 s elongation at 72° C in a model HBTR1 thermal reactor (Hybaid, Middlesex, UK). Reagents were from the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, Conn.) and the template was pAK49 linearized with *Eco*RI. For the construction of pKR53 and pKR54, pMP101 linearized with *Eco*RV was used as the template.

DNA sequence analysis

Sequencing from the ends of γδ into the flanking *Lactococcus* DNA was accomplished with primers corresponding to nucleotides 5899–5919 (primer γδ-1; GGGGAACTGAGAGCTCTAAAT) and complementary to nucleotides 84–104 (primer γδ-2; CAACATACGAAAGGGTCCCTG) of γδ (Genbank/EMBL accession number X60200). Sequencing was with the dsDNA Cycle Sequencing System (BRL Life Technologies, Gaithersburg, Md.) following the recommended procedure. Reactions were also done with primer 1 on pAK49. Computer analysis of the DNA sequences and derived amino acid sequences was with the GCG software package version 7.1 (UNIX) (Devereux et al. 1984).

Test for single-stranded DNA

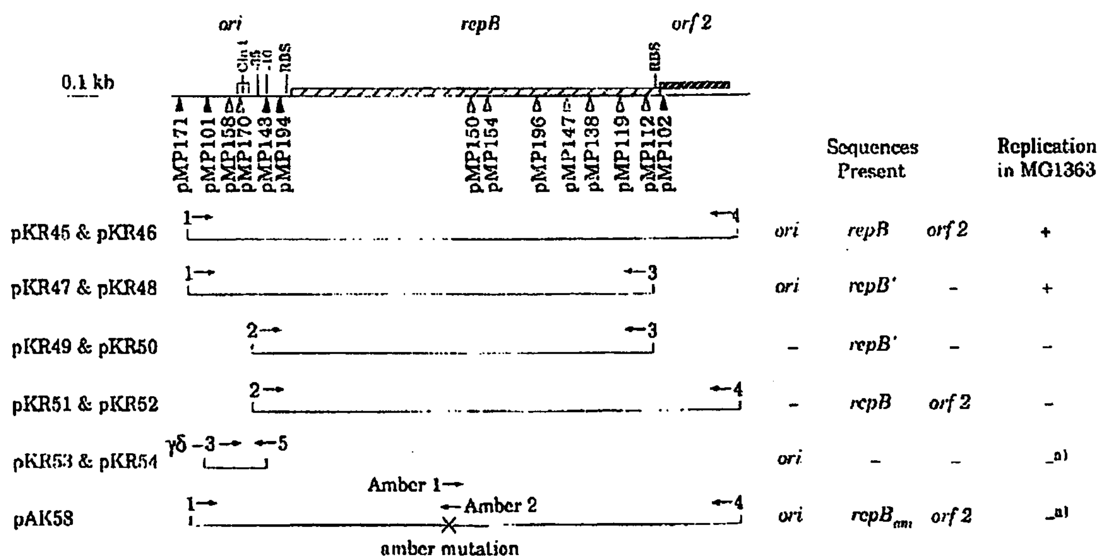
Total genomic *Lactococcus* DNA was prepared using the method of Johansen and Kibenich (1992a), digested with *Xba*I and fractionated in an agarose gel. For denaturation, the gel was incubated 30 min in 0.5 *N* NaOH, 1.5 M NaCl then neutralized 30 min in 0.5 M TRIS, 1.5 M NaCl (pH 7.5). The undenatured gel was incubated in H_2O instead of NaOH, NaCl. DNA was transferred to GeneScreen Plus filters (E.I. du Pont de Nemours, Boston, Mass.) by capillary action. Labelling of probe DNA and hybridization was with the Enhanced Chemiluminescence gene detection system of Amersham (Buckinghamshire, UK) following the manufacturer's recommended procedure.

Results

Construction of pAK49

DB1138 contains five plasmids including pCT1138, the 5.5 MDa citrate plasmid. The citrate plasmid was cloned into pVA891 (Ery[R]) using the unique *Bam*HI site located in the citrate permease gene (David et al. 1990) to give pAK49. Restriction mapping of pAK49 with

Fig. 1 Physical and genetic map of the replication region of the citrate plasmid from DB1138. *Triangles* indicate the site of γδ insertions; *open triangles* represent insertions blocking replication, *closed triangles* insertions allowing replication in MG1363. *Lines* indicate the sequences present in the polymerase chain reaction (PCR) subclones; *numbers* and *arrows* indicate the primers used in the PCR reactions. Pairs of plasmids differ only in the orientation of the *Hin*dIII fragment inserted in pVA891. Replication of these plasmids in MG1363 is designated +, the ability to replicate −, the ability to replicate if *repB* is provided *in trans* −[a]. *repB'* is truncated *repB*, lacking 29 bp



```
                              amber
amber 1            5' AAA CTC TAG AGC AAG TAT TCG  3'
                      ||| ||| *|* ||| ||| ||| |||
 5' ... GAA CTA CAA AAA CTC AAT AGC AAG TAT TCG ATT ...   3'

RepB ... glu leu gln lys leu asn ser lys tyr ser ile ...

 3' ... CTT GAT GTT TTT GAG TTA TCG TTC ATA AGC TAA ...   5'
            ||| ||| ||| ||| *|* ||| |||
amber 2  3' GAT GTT TTT GAG ATC TCG TTC  5'
```

Fig. 2 Introduction of an amber mutation in *repB*. The primers, amber 1 and amber 2, are above and below the *repB* sequence. The mismatched base pairs are indicated by *. The *Xba*I site introduced is underlined and the amber codon (TAG) introduced at codon 159 of *repB* indicated

*Bgl*II, *Pst*I, *Xba*I, *Cla*I, *Eco*RI and *Eco*RV revealed no differences between pCT1138 and the published map of pCT176 (Gasson and Davies 1984). Transformation of MG1363 with pAK49 was efficient (>10^5 Ery^R transformants/µg DNA), yielding transformants containing only pAK49. Digestion of pAK49 purified from MG1363 with *Bcl*I produced three bands, as expected from the published maps, indicating that *Lactococcus* does not have *dam* methylation.

γδ mutagenesis of pAK49

F-factor mediated transfer of plasmids lacking a Mob site from one cell to another involves cointegrate formation, facilitated by transposon γδ resident on the F-factor. Resolution of this cointegrate results in duplication of the transposon with the second copy being in the transferred plasmid (Berg et al. 1989). The approximate location of γδ in 100 pAK49 mutants from five independent matings (pMP101–pMP200) was determined by digestion with *Bam*HI and *Hin*dIII. Mutants with insertions in the region of interest were further mapped. The precise locations of γδ in 14 mutants were determined by DNA sequencing (Fig. 1). Electroporation of MG1363 with these 14 mutant plasmids was carried out and the results are presented in Fig. 1.

PCR subcloning of the replication region

Primers were designed to allow the production of clones containing various components of the replication region of pCT1138. After PCR reactions, fragments were cloned into the *Eco*RI site of pIC19H using the *Eco*RI site at the 5' end of each primer. The pIC19H polylinker contains a variety of restriction sites flanked by *Hin*dIII sites (Marsh et al. 1984). After mapping, the fragments were moved to pVA891 as *Hin*dIII fragments. Both orientations of the inserted *Hin*dIII fragment were obtained in each case. These plasmids were designated pKR45–pKR54. The structures of the various fragments and the results of electroporation of MG1363 are shown in Fig. 1.

Introduction of an amber mutation into *repB*

Two mismatch primers that would introduce an amber mutation into repB and simultaneously create an *Xba*I site were designed (Fig. 2). PCR was done with primer 1 and amber 2 and with primer 4 and amber 1, giving fragments of 0.8 and 0.9 kb, respectively. These were digested with *Eco*RI and *Xba*I, mixed and cloned in *Eco*RI-digested pIC19H. Fifteen of 18 clones analyzed had both fragments, joined at the *Xba*I site to give a 1.7 kb *Eco*RI fragment. The insert from one such clone was moved to pVA891 as a *Hin*dIII fragment, producing pAK58 which is identical to pKR46 but contains an amber mutation in *repB* (Fig. 1).

Electroporation of MG1363 with a mixture of pNZ18 (Cam^R), and pAK58 (Ery^R) produced Cam^R transformants but no Ery^R transformants. Electroporation of the nonsense suppressor strain FD100 with the same plasmid mixture produced both Cam^R and Ery^R colonies. Analysis of these transformants revealed that 25% of the Cam^R colonies were also Ery^R. *Xba*I digestion of plasmids purified from FD100 confirmed that pAK58 still contains the amber mutation. Thus, the amber mutation in *repB* can be suppressed by the ochre suppressor in FD100 but is not complemented by pNZ18.

Complementation of the amber mutation in pAK58

Plasmid pKR44 is pIC19H containing the PCR product produced with primer 2 and primer 4 and is the source of the *Hin*dIII fragment cloned into pVA891 to produce pKR51 and pKR52 (Fig. 1). The insert in pKR44 contains *repB* and was cloned, as a *Cla*I-*Eco*RI fragment, into the shuttle vector pCI3340 (Cam^R). The resulting plasmid, pMP44, contains the same fragment as that inserted in pKR51 and pKR52 except for a few base pairs of pIC19H polylinker.

Electroporations were done with mixtures of pAK58 and pMP44 or pAK58 and pCI3340 selecting for Cam^R and Ery^R. When pAK58 was mixed with pCI3340, colonies appeared on chloramphenicol but not on erythromycin plates. Thus, pCI3340 cannot complement the amber mutation in *repB*. When pAK58 was mixed with pMP44, colonies appeared on both chloramphenicol and erythromycin plates at a frequency of approximately 10^5/µg DNA. Analysis of plasmids from Ery^R

```
         1                                                                                                        100
pCT1138  CCTATTATAT ATTTATCATA TATATTTTAA TCTTTTGTTC TTTTGCGTGA AAAAAAAGGC AGTGTTTTCG CTAGTTATAG AAATTAAACA GTCACAAAAA

pSL2     CCTATTATAT ATTTATCATA TATATTTTAA TCTTTTGTTC TTTTGCGTGA AAAAAAAGGC AGTGTTTTCG CTAGTTATAG AAATTAAACA GTCACAAAAA
         672                                                                                                      771

         101                                                                                                           181
pCT1138  TCGATGTATA GAGTCACAAA A......... .......... ...ATCGATG TATA.GAGTC ACAAAAATCG ATGTACACAG CACGACTTTT GTATTTGTGT ACTG
                    *            ********* ********** ***                *                       *
pSL2     TCGATGTATA CAGTCACAAA AATCGATTTT TGTGACTCTA TGCATCGATG TATACGAGTC ACAAAAATCG ATGTACACAG TACGACTTTT GTATTTGTGT ACTG
         772                                                                                                           875
```

Fig. 3 Nucleotide sequence of the pCT1138 replication origin and comparison to pSL2 (Jahns *et al.* 1991). Differences are indicated by *. The GenBank/EMBL accession number for the nucleotide sequence of this region of pCT1138 is L27067

```
         1 MP101                                                                           MP158                 100
pCT1138  CCTATTATAT ATTTATCATA TATATTTTAA TCTTTTGTTC TTTTGCGTGA AAAAAAAGGC AGTGTTTTCG CTAGTTATAG AAATTAAACA GTCACAAAAA
             ----------->   ----------->                                                                 -----------------
pCI305   CCTATTATAT ATTTATCTTA TATATTTTAA TCTTTTATTC TTTTGCGTCA AAAAAAAATC AATATTTTCA AGGCTTTATA GAATTATATA CCAACAAAAA
             ----------->   ----------->                                                                 -----------------
pCI528   CCTA.TATTA ATTTATCATA TATATTTTAA TCTTTTCTTC TTTTGCGTCA AAAAAAAGTT AGTATTTTTA AGGGGTTACA GAATAATATA GCATAAAAAA
             ----------->   ----------->                                                                 -----------------
pSK11L   CTTTTAATTA TTTTATATTA TATATTTT.G TCTTTTGTTC TTTTGCGAAA AAAAAAATCT AGTGTTTGCA AGGGGTAACA GGATTATAGT CCTACAAAAA
             ----------->   ------------>                                                                -----------------
pVS40    CCTA.TATTA ATTTATCATA TATATTTTAA TCTTTTATTC TTTTGCGTGG GAAAAAAGTC AGTGTTTAAA GGTGGATACA GAATTATAGC GTATGAAAAA
             ----------->   ------------>                                                                -----------------
pWV02    CCTA.TATCT ATTTATCATA TATATTTTAA TCTTTTA.TC TTTTGCGTGA AAAAAAAGTC AGTGGTAGCA AGGGTATGCA GAATTAAACA GTCAGAAAAA
             ----------->   ----------->                                                                 -----------------

         101        MP170     +          +          *                     -35                 MP143      -10  197
pCT1138  TCGATGTATA GAGTCACAAA AATCGATGTA TAGAGTCACA AAAATCGATG TACACAGCAC GACTTTTGTA TTTGTGTACT GTATATA... GTATAATAAA
         ----->----------------------->----------------------->
pCI305   ACTGTGTATA TACCAACAAA AAACTGTGCA TACACCAACA AAAAACTGTG CATATACCAA CTTCTTTGTT TGTTTCGTTG GTATATAATG ATATAATAAA
         ----->----------------------->-----------------------> ----------->
pCI528   ACTGTGTATA TAGCATAAAA AAACTGTGTA TATAGCATAA AAAAACTGTG TATATAGCAT AAAAAAATCA TCATTTTATG CTATATTATG ATATAATAAA
         ----->----------------------->-----------------------> ----------------->
pSK11L   ACTGTGCATA GTCCTACAAA AAACTGTGTA TAGTCCTACA AAAAACTGTG TATAGTCCTA CAAGTTATTT GTGTTTGTAG GTGTTTCGTG TTATTATTTA
         ----->----------------------->-----------------------> ------------->
pVS40    ACTGTGTATA GCGTATGAAA AAACTGTGTA TAGCGTATGA AAAAACTGTG TATAGCGTAT GAAAAAACTT GATAGATATA CGGTATTCTG ATACAATAAA
         ----->----------------------->-----------------------> ----------------------->
pWV02    ACTGTGTATA CAGTCAGAAA AAACTGTGTA TACAGTCAGA AAAAACTGTG TATACAGTCA GAAAAAATTG AACAACTGAC TGCGTTTTGA TATAATAAAA
         ----->----------------------->-----------------------> ----------------->

         198                                    MP 194                           275
pCT1138  AGCATAGAGA AAACTCACTA TGAAATGACT TTCTCTATGC TACTACTAAA ACACGCAAAG GAGCGTATTT ATACTATG.
pCI305   AGCAT.GAAG AATCTCTCTA CGAAAAGTGT TTCTTCATGC TTATCTAAAC TCACTCACAA AGGAGCAGTT TTCTATG..
pCI528   AGTATGAAGA ACAAACTTTT GAACGAGAAT TTCTTCATAC TTACTTATGA ACACGCAGAG GAGCGTATCT TTATG....
pSK11L   TTTAAATCAT AAAAGGAGTG GATTATG... .......... .......... .......... .......... ........
pVS40    AGCATAGAGA AATCGACAGC GAA.ATCAGT TTCTCTATGC CTACTTAAAA TCACTCACAA AGGAGTAACT TTCTATG..
pWV02    AGCATAGAGA AATTGACTCG CTAAAGATTT TTCTCTATGC CTATTTAAAA ACACTCACAA AGGAGTATTA CTATG....
```

Fig. 4 Comparison of the replication origins of six *Lactococcus* plasmids. Nucleotide numbers above the sequences are for pCT1138. All sequences end with the start codon for the Rep protein. *Arrows* indicate the small direct repeats and tandem repeats. The −35 and −10 regions of the potential promoters are *underlined*. Insertions of γδ in this region are indicated by the plasmid name (prefix MP) above the 5 bp duplicated upon integration of the transposon. The 5′ end of the citrate plasmid sequence included in PCR primer 2 is indicated with *. The locations of the additional base pairs in pSL2 are indicated with +

transformants revealed that all 20 of the transformants tested had both pAK58 and pMP44 and that the amber mutation was still present in pAK58. From this, we conclude that pMP44 produces an active RepB protein; that this protein acts *in trans* to allow replication of pAK58; and that the inability of pKR51 and pKR52 to replicate in MG1363 is not due to the introduction of mutations in *repB* by errors in PCR.

Cloning of the citrate plasmid replication origin

PCR was carried out on pMP101 DNA with primers γδ-3 and primer 5. This allowed amplification of a fragment containing 25 bp of γδ DNA and 179 bp of citrate plasmid DNA with *Eco*RI sites at both ends. This fragment was cloned into pIC19H to produce pAK107 and then moved into pVA891, as described above, to produce pKR53 and pKR54, which differ only in the orientation of the *Hin*dIII fragment inserted in pVA891.

The DNA sequence of the insert in pAK107 was determined using the direct and reverse sequencing primers of Boehringer-Mannheim (Mannheim, Germany). No PCR-induced alterations were detected. The sequence of the pCT1138 DNA on this fragment and a comparison with the corresponding sequence from

```
          1                                                                                                              99
pCT1138   .MIIPEKONK QKQVLTLNEL EKRKVVEHNA LIQSVAKNQK TALKNFELAV SCIDTEEPPK NNTVYLLKSE LFKFFEVSSS SKHSQFKEAV NYNQKQAFFN
pCI305    MSSISKNEPN QKQVQTLNEL SKRKVVEHNS LITSIAKNDK TPLKNFELAV SCINTEEPPK DHTVYLSKEE LFAFFKVSDN DKHSRFKQAV ENNQKQAFFQ
pCI528    MSIITEFEKN QKQVKALNEL SKRKVVEHNS LITSIAKNDK TPLKNFELAV SCINTEAPPK DHTVYLSKTE LFAFFKVSDN DKHSRFKQAV ENNQKQAFFK
pSK11L    MQKIDTGERN KQ....IQEI SSRKVAEHND LISSVAKNDK TPLKNFELAV SCIDTDAPPK DNIVFLSKKE LFTFFDVSDN DKHRRFKEAV EKNQEQAFFR
pVS40     MSIIPEKQEN QKQVLTLNEL SKRKVVEHNS LITSIAKNDK TPLKNFELAV SCINTEEPPK DNTVYLSKRD LFAFFKVSDN DKHSRFKQAV EKNQKQAFFQ
pWV02     MPSIAEKQEN QKQVLTLNEL SKRKVVEHNS LITSIAKNDK TPLKNFELAV SCINTEEPPK DNAVYLSKEE LFTFFKVDDN DKHSRFKQAV AKNQEQAFFE
consensus m..i.....n qkqv..lnEl skRKVvEHN. LI.S.AKNdK TpLKNFELAV SCI.Te.PPK d.tVyLsK.e LF.FF.Vsdn dKHsrFKqAV ..NQ.QAFF.

          100                                                    α                                               199
pCT1138   IKADKKLGIE YESIVPIPYV KMNDYNDEVT IRFDQAINPY LIDLKAEFTQ YKISELQKLN SKYSIILYRW LSNNYNQYEH YSVKGGRRVE QVESYRNPSI
pCI305    IKEEVGKGFK FRSIVPIPYV EWTDYHDDVK IEFHREINPY LINLKQNFTQ HALSDIAELN SKYSIILYRW LSNNYNQYEH YSYKGGRREE QVEAYRNPTI
pCI528    IQEKKEYGFE FENIVPIPYV KWADYHDEVT IRFSPEINPY LINLKQNFTQ HALSDIAELN SKYSIILYRW LSNNYNQYEH YSAKGGRREE QVETYRNPPI
pSK11L    IKEKKNRGFK FKRIVPIPYV EWNDYNDKVL IRFDQAINPY LIDLKNNFTQ YAISDIKELN SKYSIILYKW FSNSYNQFEH YQYKPNRTKK QLEDYKSPRI
pVS40     IKEEQGKGFK FKSIVPIPYV EWTDYNDEVK IEFHREINPY LINLKKNFTQ HALSDIAELN SKYSIILYRW LSNNYNQYEH YSVKGGRRAE QVEAYRNPSI
pWV02     IKEEKNKGFK FRRILPIPTV EWTDYDDKVN IRFNQDINPY LIELNENFSK YALSEIKELN SKYSIILYKW LSNNYNQYEH YSNKGGRRAE QVESYRDPSI
consensus Ike....Gf. ...IvPIPyV .W.DY.D.V. I.F...INPY LI.Lk.nFtq .a.S.i.eLN SKYSIILY.W lSNnYNQyEH Ys.KggRr.e QvE.Yr.P.I

          200                                                                                                    299
pCT1138   KVKELREITD TIKEHQHFPH FETRVLKKAI EEINAHTSFN VTYEKKKAGR SIDSIVFNIE KKRNADDNSY KLGDKDYQDD KKQKSRNEAD LLKQANESKY
pCI305    SNRELRENTD TVDEYPRFDR LEHRVLKEPI EEINENTSFN VTYDKIKKGR SIDSIVFNIT KKRRADDNSY KLEDKDYQSD KEEKSRNEAD LLKQANESKY
pCI528    SIRELRENTD TNKDYPRFQS LESYIIKNSL KEINEHTSFK VTYEKVKKGR SINSIVFNIT KKRRADDNSY KLEDKVYQKA KVQKEQKENL LYAEANQSKY
pSK11L    IISDLRELTD TVDDYSRFDN FEKRVIKDAI KEINSFTHFN VEYKKIKKGR SIDSIQFNIV KKANVKDENY KRNDVQAQLT EEQNQAQNQV NYAVAVANPF
pVS40     SIKELRIITD TVNSYKQFTR FNKDVLKTPL DEINANTSFN VTYDKIKKGR SIDSIVFNIE KKRNADDNSY KLDDRAYQED KARKAETEDQ LVLQANDSPY
pWV02     SVKELRTITD TVNEYRHFPH FEKWILKKPL EEINAHTSFN VSYEKIKKGR SIDSIVFNIE KKRKADDNSY KLEDQTYIEG KKAKEETEKD LYTESNQSPY
consensus ..keLR..TD T...y..F.. ......K... .EIN..TsFn V.Y.K.KkGR SIdSIvFNI. KKr.adDnsY Kl.D..yq.. k..k...e.. l...an.s.y

          300                                                      *                    + Δ       385
pCT1138   TRLLSENFLI GNRDINDTAT NVGLQKNVYP LYDELKELRG LNGVKDHLSY VASKKEAYS. .KRNVAKYLK KAIEQYL.PT VKRQDLNNE. .......... ..
pCI305    TRLLIENFLL SPLENTDTAL NAGLQKNVYP LYDELKELRG LNGVKDHLSY ISSKKEAYS. .KRNVAKYLK KAIEQYL.PT VKRQDLNNE. .......... ..
pCI528    TKLLLENFLL SPYENTNPAT NAGLQRNVYP KYDELKDLNG IDGVKKHLSY IYDKQEPYS. .KGNIAKYLK KAIEQYL.PT VKRRGL.... .......... ..
pSK11L    TNKLINSSLL YATDIANQDT ILELAESVHP VYDKLVKELG EDALETHNDY VRRKNVDYSN DKKNIVKYLS ISAKQYLNPR LSKQQNKE.. .......... ..
pVS40     TKLLNENFLL SYLDLNDKKI LAGLQKNVYP LYDELKDLRG LNGVKDHLSY VASKKEAYS. .KRNVAKYLK KAIEQYL.PT VKLQDLEQPE RAKVRGKGAS NE
pWV02     TKLLSENNLL FPNDFNDIKT NAGLQKNVYP LYDELKDLRG LNGVKDHLSY VSSKQEDYS. .KRNVAKYLK TAIEQYL.PT VKRQDL.... .......... ..
consensus T.lL.e..Ll .......... ..gLq.nVyP .YDeLk.l.G ..gvk.HlsY ...K.e.YS. .K.N.aKYLk .aieQYL.Pt vk.q.l.... .......... ..
```

Fig. 5 Alignment of the amino acid sequence of the Rep protein of six *Lactococcus* plasmids. Amino acid numbers above the sequences are for pCT1138. In the consensus, *upper case letters* are for amino acids conserved in all six plasmids, *lower case letters* represent amino acids conserved in five of the plasmids. The point of divergence of pCT1138 with the published sequence (Jahns et al. 1991) is marked *; the divergence caused by γδ in pMP112, +; the divergence caused by the deletion in pKR47–pKR50, Δ; and the asparagine residue replaced by an amber codon in pAK58, α

pSL2 is shown in Fig. 3. The first 2 bp actually derive from γδ in the PCR clone, but are identical to those normally present at this location in the citrate plasmid (Fig. 4).

The ability of this fragment to allow replication was tested by electroporation of MG1363 with a mixture of pKR53 or pKR54 (Ery^R) and pMP44 or pCI3340 (Cam^R). When pKR53 or pKR54 was mixed with pCI3340, colonies appeared on chloramphenicol but not on erythromycin plates, indicating that pKR53 and pKR54 cannot replicate alone or in the presence of pCI3340. When pKR53 or pKR54 was mixed with pMP44, colonies appeared on both chloramphenicol and erythromycin plates at a frequency of approximately 10^5/μg DNA. Plasmid analysis revealed that the Ery^R isolates contained both pKR53 or pKR54 and pMP44. Thus, when the appropriate RepB protein is provided from another replicon, the citrate plasmid sequence presented in Fig. 3 is sufficient to allow plasmid replication in MG1363. We conclude that this sequence includes the origin of replication of pCT1138.

DNA sequence analysis

Sequencing from γδ into the flanking *Lactococcus* plasmid DNA revealed significant differences from the published sequence (Jahns et al. 1991) in two regions. Nucleotides 793–814 and 826 of the published sequence are absent in pCT1138. Also, two single base-pair differences were found (Fig. 3). This region was sequenced on both strands from three insertion mutants, using the transposon primers and pAK49 with PCR primer 1. Nucleotides 2013–2017 are five A residues according to Jahns et al. (1991). We find however that there are actually six A residues in this region. This frameshift extends *repB* and results in the amino acid sequence indicated in Fig. 5. This difference was seen in mutant as well as wild-type plasmids.

Test for single-stranded DNA

Filters containing denatured and undenatured total genomic DNA from DB1138, MG1363 and MG1363/pNZ18 were probed with a mixture of pAK58 and pNZ18 (Fig. 6). No hybridization to MG1363 was seen. Hybridization to the 1.6 kb size standard was due to homology to the pVA891 part of pAK58 (not shown). MG1363/pNZ18 gave a single band when not denatured and multiple bands when denatured. The band common to both hybridizations must be the single-stranded pNZ18 replication intermediate, while the major band seen upon denaturation is linearized double-stranded pNZ18. Identical results were obtained when

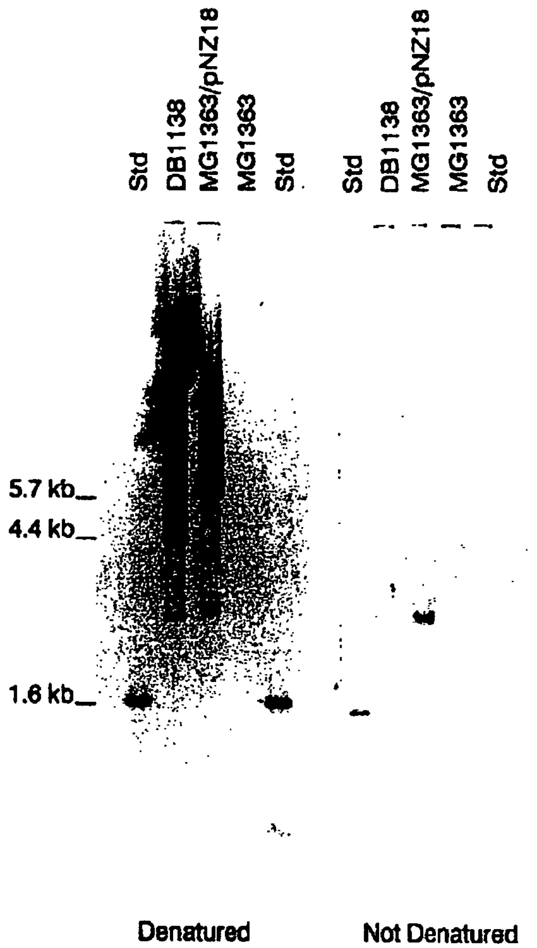


Fig. 6 Hybridization of denatured and undenatured total genomic DNA with a mixture of pNZ18 and pAK58. Sizes are estimated from a 1 kb ladder size standard

pNZ18 alone was used as the probe (not shown). Hybridization to DB1138 was only detected when the DNA was denatured; no hybridization to undenatured DB1138 occurred. The major band is the 4.4 kb *Xba*I fragment containing the citrate plasmid replication region; the remaining hybridization could be to the replication region of other plasmids in DB1138. In any case, under conditions where the single-stranded replication intermediate of pNZ18 was detectable, single-stranded replication intermediates of the citrate plasmid could not be detected.

Discussion

We have genetically analyzed the replication region of pCT1138, the citrate plasmid of *L. lactis* subsp. *lactis* biovar *diacetylactis* strain DB1138 with the goal of defining its essential features. The DNA sequence of the corresponding region from pSL2, the citrate plasmid of strain Bu2, has been published (Jahns et al. 1991). Significant differences from this sequence were observed in two regions. One difference affects the structure of the putative origin of replication. The other difference results in an increase in the size of the potential protein coded by *repB* and increases the homology to similar proteins. No additional differences were detected in this region (bp 1360–2280 of Jahns et al. 1991).

Common features of the replication origins of pSK11L and pCI305 have been described (Horng et al. 1991). Both plasmids contain a pair of short direct repeats containing only A and T residues, separated by 3 bp. These repeats are 10 bp long in pCI305 and 11 bp in length in pSK11L. As illustrated in Fig. 4, pCT1138 has 10 bp imperfect repeats (one mismatch) separated by 3 bp. Both plasmids have a 22 bp sequence tandemly repeated 3.5 times. The citrate plasmid of DB1138 has a 22 bp sequence repeated 3 times. The distance between the small direct repeats and the 22 bp tandem repeats is 55 bp in pSK11L and 57 bp in pCI305 and pCT1138. This interval contains the sequence TCTTTT repeated twice and a string of A residues. Insertion of $\gamma\delta$ into this 57 bp interval in plasmid pMP158 (Fig. 4) prevents replication, presumably by destroying an essential structure or increasing the length of this interval to over 6 kb. Insertion into one of the 22 bp tandem repeats in plasmid pMP170 also prevents replication in MG1363, as does elimination of the tandem repeats and upstream material in pKR51 and pKR52. Insertion immediately upstream of the short direct repeats (pMP101) has no effect, delineating the left end of this structure. The insertion in pMP143, downstream of the tandem repeats, does not affect replication, delineating the right end of this structure. Thus, the region between the $\gamma\delta$ insertions in pMP101 and pMP143 is essential for replication in MG1363 and probably represents the origin of replication. This was confirmed by demonstrating that this region, in clones pKR53 and pKR54, was sufficient to allow plasmid replication when the citrate plasmid *repB* gene was provided on another replicon (pMP44). A similar origin structure exists in plasmids pVS40 (von Wright and Räty 1993), pWVO2 (Kiewiet et al. 1993a) and pCI528 (Lucey et al. 1993). These sequences are aligned in Fig. 4. None of these plasmids contain the 23 additional base pairs found in pSL2 (Fig. 3), suggesting that pSL2 is atypical.

Jahns et al. (1991) cloned the Ery^R gene of pVS2 into the *Cla*I site of the citrate plasmid, pSL2, to construct a plasmid designated pSL2E2. Deletion derivatives of pSL2E2 were made *in vitro* and at least one of these was found to replicate in *Lactococcus*. The 22 bp tandem repeat sequence of the citrate plasmid contains a *Cla*I site so the construction of pSL2E2 should have destroyed the replication origin by eliminating all but one copy of the repeat sequence. Subsequent deletions would remove most of this remaining repeat sequence, the small direct repeats and the intervening 57 bp. Since

DNA sequence data is not presented for these derivatives, it is impossible to reconcile this observation with our finding that this region is essential for replication in MG1363.

Immediately downstream from the tandem repeats is the coding region for *repB* and its promoter. A γδ insertion between the −35 and the −10 region of this promoter in pMP143 has no effect on replication. Perhaps the transposon sequence TTGGCC, 10 bp from the end of γδ and 17 bp from the −10 region, is an acceptable −35 region. Outward facing −35 regions on transposable elements have been described previously (Galas and Chandler 1989), and also in lactic acid bacteria (Johansen and Kibenich 1992b). The insertion in pMP194 separates the promoter from the coding region, yet has no effect on replication. Possibly the −35 region on the transposon combined with the sequence TAAAAC 18 bp downstream produces a functional promoter. Alternatively, a sequence on γδ may act as a promoter in *Lactococcus*.

repB codes for a protein highly homologous to the RepB protein of pCI305 (Hayes et al. 1991) and pWVO2 (Kiewiet et al. 1993a), the Rep protein of pSK11L (Horng et al. 1991) and the putative replication protein of pCI528 (Lucey et al. 1993) and pVS40 (von Wright and Räty 1993). These sequences are aligned in Fig. 5.

Mutations in *repB* prevent replication in MG1363 (Fig. 1), indicating this gene to be essential. An amber mutation in *repB* blocks replication in MG1363 but not in an ochre suppressor strain. Thus translation of *repB* is necessary for plasmid replication. The RepB protein of the citrate plasmid, provided *in trans* by plasmid pMP44, complements the defect in pAK58. The RepB protein of pCI305, provided *in trans* by pCI3340, does not complement the amber mutation in pAK58 in spite of the structural similarity. This specificity indicates that the differences in the sequences of the various replication origins and the RepB proteins are significant.

Since the PCR primers were designed based on the published sequence, primer 3 actually lies within the *repB* coding region. Amplification using this primer will give clones in which the carboxy-terminal ten amino acids of the RepB protein have been substituted by five amino acids coded for by the pIC19H polylinker. Clones pKR47 and pKR48 contain such fragments and replicate in MG1363. These plasmids are, however, readily lost in the absence of antibiotic selection and have a reduced copy number compared to clones with the complete RepB protein (e.g. pMP102, pKR45 and pKR46, data not shown). The insertion in pMP112 prevents replication and causes substitution of two γδ-encoded amino acids for 13 carboxy-terminus amino acids. The difference between the effects these two mutations have on the ability to replicate could be due to the resulting amino acid sequence or the size of the resulting protein.

No homology between the potential Orf 2 protein and the protein sequence data bank was detected. Insertions in Orf 2 in plasmid pMP102 or deletion of the entire open reading frame in plasmids pKR47 and pKR48 did not prevent replication. Thus, Orf 2 is not essential for replication of the citrate plasmid in MG1363.

Based on our data, the complete minimal replicon for the citrate plasmid of DB1138 extends from nucleotide 672 to 2190 (Jahns et al. 1991) and contains the origin of replication, a promoter and a single open reading frame, *repB*. The carboxy-terminus 29 bp of *repB* can be deleted, producing a plasmid replicating with reduced copy number.

Lactococcus plasmids are divided into two groups based on their mode of replication. One group replicates by the rolling-circle method and is characterized by the accumulation of single-stranded DNA intermediates (Gruss and Ehrlich 1989). The other group does not accumulate single-stranded DNA and replicates by theta replication (Hayes et al. 1991; von Wright and Räty 1993, Kiewiet et al. 1993a). No single-stranded DNA could be detected in DB1138 when the citrate plasmid replication region was used as a probe. Similar results were obtained for three other plasmids with replication regions similar to that of the citrate plasmid (pCI305, Hayes et al. 1991; pVS40, von Wright and Räty 1993; pWVO2, Kiewiet et al 1993a). These results, combined with the lack of significant homology between the citrate plasmid replication region and plasmids utilizing the rolling-circle method of replication (e.g. pWVO1, Leenhouts et al. 1991; pC194, Gros et al. 1987), suggest that the citrate plasmid replicates by theta replication.

Recombinant plasmids using theta replication have higher structural stability in *Lactococcus* than those using rolling-circle replication (Kiewiet et al. 1993b). Thus, the citrate plasmid replication region is potentially useful for the construction of food-grade cloning vectors. The knowledge, provided here, of the essential elements in this replicon will help in these constructions.

Acknowledgements We are grateful to Annette Kibenich for excellent technical assistance; Egon Bech Hansen for advice on cycle sequencing and γδ mutagenesis, helpful comments and careful reading of the manuscript; and Michael Blom Sørensen (Carlsberg Laboratories) for synthesizing primers. We thank Atte von Wright for communicating the sequence of the replication region of pVS40 prior to publication and Françoise Dickely for providing the nonsense suppressor strain FD100. This work was supported by a grant from the Lundbeck Foundation.

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Date of mailing (day/month/year) 12 May 1999 (12.05.99)	
Applicant's or agent's file reference 21030 PC 1	**IMPORTANT NOTIFICATION**
International application No. PCT/DK99/00209	International filing date (day/month/year) 14 April 1999 (14.04.99)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 21 April 1998 (21.04.98)
Applicant CHR. HANSEN A/S et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

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Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
21 Apri 1998 (21.04.98)	0551/98	DK	11 May 1999 (11.05.99)
21 Apri 1998 (21.04.98)	60/082,555	US	27 Apri 1999 (27.04.99)

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